USF1 promotes the development of knee osteoarthritis by activating the NF-κB signaling pathway

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Abstract. The current study mainly aims to evaluate the expression pattern and underlying mechanism of upstream stimulating factor 1 (USF1) in the muscle tissues of knee osteoarthritis (KOA) patients. In accordance with previous findings, our data showed that muscle strength was significantly decreased in KOA patients compared with controls. Furthermore, several inflammatory factors, including tumor necrosis factor α (TNFα), IL-8, IL-6 and MCP-1, were associated with reduced muscle strength in KOA patients. Not surprisingly, NF-κB signaling was significantly activated in the muscle tissues of KOA patients compared with control individuals. Furthermore, we showed that USF1 was increased in the muscles of KOA patients compared with controls. More importantly, overexpression of USF1 in primary human skeletal muscle cells significantly increased the activation of NF-κB signaling as well as the levels of pro-inflammatory factors. In summary, we showed novel data that the upregulation of USF1 promoted NF-κB activation-induced inflammatory responses in muscle tissues of KOA patients.

Introduction

Knee osteoarthritis (KOA) is a major cause of disability among the elderly (1). Inflammation of the synovial joint plays a key role in the progression of KOA and also results in pain and disability (2). Furthermore, increased inflammation and pain can decrease the use of the knee extensor muscles thereby relieving the joint load related to knee function (3,4). Thus, it is common for muscle atrophy to occur beginning at the onset of knee OA (5). It has been well established that the knee extensor muscles mainly function as a key regulators in the maintenance of daily walking activities (6). However, the underlying mechanism by which inflammation is modulated in the skeletal muscles (knee extensors) of KOA patients is poorly understood.

In response to insult and/or injury, inflammation is induced and participates in cell injury (7). Abnormal expression of inflammatory factors, including interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), has been widely reported in KOA patients (8). Within muscle, p65 NF-κB signaling is one of the key signaling pathways that upregulates cytokine gene expression, including TNFα, IL-1β, IL-6, and MCP-1 (7,9). Undoubtedly, activation of p65 NF-κB signaling in muscle may decrease muscle strength and function (10,11).Upstream stimulating factor 1 (USF1), which is a 43-kDa protein, is a key member of the eukaryotic evolutionarily conserved basic helix-loop-helix-leucine zipper transcription factor family (12). USF1 has been reported to be involved in multiple biological processes, including cell proliferation and lipogenesis, by binding the E-box regulatory elements (CANNTG) (13-15). The mechanism of the problem of muscle movement caused by KOA is not clear. Previous studies have shown that upstream stimulatory factor (USF1) plays a key role in various muscle cells. For instance, USF1 is shown to regulate human cGMP-dependent protein kinase I gene expression in vascular smooth muscle cells, thereby maintaining smooth muscle cell relaxation, growth, and differentiation (16). Furthermore, USF1 is demonstrated to modulate the expression of osteopontin in cultured vascular smooth muscle cells and might promote initial osteopontin expression observed post carotid injury *in vivo* (17). In skeletal muscle, USF1 is shown to increase PGC-1alpha promoter activation (18). However, whether USF1 is abnormally expressed in the muscle tissues of KOA patients has never been explored.

The current study mainly aims to evaluate the expression pattern and underlying mechanism of action of USF1 in the muscle tissues of KOA patients, which may shed light on the prevention and treatment of KOA.

Materials and methods

Patient samples. In the current study, twenty patients (10 men and 10 women) with diagnosed KOA and five control individuals (3 men and 2 female) were recruited from Hongqi

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Hospital Affiliated with Mudanjiang Medical University. These patients were scheduled for knee replacement surgery and able to walk at least forty-five meters independently (without the use of walking aids). Patients were excluded if they had uncontrolled systemic disease (non-musculoskeletal conditions that would make testing difficult and uncomfortable for the participants, such as chronic obstructive airway disease or congestive heart failure) or a preexisting neurologic or other orthopedic condition affecting walking. The study protocol was approved by the Human Research Ethics Committees of Hongqi Hospital Affiliated with Mudanjiang Medical University. All of the participants were informed about the nature of the study and signed a consent form prior to participation. The details for all participants are listed in Table I.

Table I. Basic physical characteristics of KOA patients and healthy controls.

Characteristics	Control	KOA	P-value
Age (years)	67.8±5.6	65.8±9.4	>0.05
Height (cm)	168.3±8.7	171.3±10.9	>0.05
Weight (kg)	67.88±20.33	73.24±8.7	>0.05
BMI (kg/m^2)	27.6±1.3	28.9±2.4	>0.05
Muscle strength (Nm)	143.5±26.5	83.5±11.5	<0.001

BMI, body mass index; KOA, knee osteoarthritis.

Cell culture. Primary human skeletal muscle cells were purchased from Procell (CP-H095, Wuhan, China, http://www.procell.com.cn/view/2244.html). The cells were cultured in specific complete medium for human skeletal muscle cells (CM-H095; Procell, Wuhan, China) supplemented with 10% heat-inactivated fetal calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 U/ml penicillin and streptomycin in 25-cm^2 culture flasks at 37˚C in a humidified atmosphere with 5% CO_2.

Determination of muscle strength. The strength of the knee extensor muscle group was determined in the affected legs of the 20 patients with KOA and in the leg from which the muscle biopsy specimen was obtained in the 7 control subjects. A portable nonextendable strain gauge (load cell) was used to measure muscle strength for this study. The strain gauge was attached to the subject's leg using a webbing strap with a Velcro fastener. The subject sat in a tall chair with a strap around the lower leg 10 cm above the ankle joint, and the hip and knee joint angles were positioned at 90 degrees. The distance from the knee joint to the strap around the ankle was measured with a tape measure and was used for the calculation of torque [force (N) xdistance (m)]. Each subject exerted maximal force against the strap assembly for 3 sec. Three trials were recorded for each subject, and the highest score was used for the analysis.

Muscle biopsy. Resting muscle samples were isolated from the vastus lateralis, as previously described (19). In brief, the muscle samples from KOA patients were collected during their knee replacement surgery ~5 cm proximal to the suprapatellar pouch. The biopsies were taken after the skin was incised and prior to knee joint capsule incision with no trauma to the muscle or the joint at that time (19).

Protein extraction and western blot analysis. Skeletal muscle (30 mg) was extracted using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and was collected following centrifugation at 12,000 x g for 30 min at 4˚C. A bicinchoninic protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration. A total of 15 μg protein was loaded per lane, separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 8% non-fat dry milk at 4˚C overnight. Following three washes with PBS with Tween 20 (5 min/wash), the membranes were incubated with the following primary antibodies at 4˚C overnight: p-p65 (#3033, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), p65 (#8242, 1:1,000; Cell Signaling Technology, Inc.), anti-IκBα (#4812, 1:1,000; Cell Signaling Technology, Inc.) USF1 (ab125020, 1:1,000; Abcam, Cambridge, MA, USA) and GAPDH (cat. no. 5174; 1:1,000; Cell Signaling Technology, Inc.). Following several washes with TBST, the membranes were incubated with horseradish-peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) or HRP-conjugated mouse antigoat IgG (ZF-0311, all 1:5,000; Zhongshan Gold Bridge Biological Technology Co., Beijing, China) for 2 h at room temperature and then washed followed by detection with enhanced chemiluminescent substrate (EMD Millipore, Billerica, MA, USA). GAPDH was used as an internal control. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for density analysis.

Adenoviral vector construction. The adenovirus vectors overexpressing USF1 (Ad-USF1) or negative control (NC) (Ad-NC) were constructed by GenChem (Shanghai, China). For the transfection of adenovirus vectors into primary human skeletal muscle cells, the cells were seeded at a density of 10^6 cells/well in 6-well plate. At 80% confluence, Ad-USF1 and Ad-NC were transfected into primary human skeletal muscle cells at 30 multiplicity of infection (MOI) for 48 h. Then, the cells were collected for further study.

Enzyme-linked immunosorbent assay (ELISA). Muscle tissue or cell lysates were centrifuged at 16,000 x g for 15 min at 4˚C, and supernatants were used to quantify the levels of TNF-α (cat no. DTA00C; Human TNF-α Quantikine ELISA kit), IL-6, (cat no. D6050; Human IL-6 Quantikine ELISA kit), IL-1β (cat no. DLB50; Human IL-1 beta/IL-1F2 Quantikine ELISA kit), and IL-8 (cat no. D8000C; Human IL-8/CXCL8 Quantikine ELISA kit) by way of a sandwich ELISA following the manufacturers' protocols (R&D Systems, Minneapolis, MN, USA). Samples were read at a 450 nm wavelength using a microplate reader (Model 3550; Thermo Fisher Scientific, Inc.).

Immunohistochemistry. Muscle tissues samples from KOA patients or control were cut into 5 μm. Then, the slices were fixed in 4% phosphate-buffered neutral formalin at room

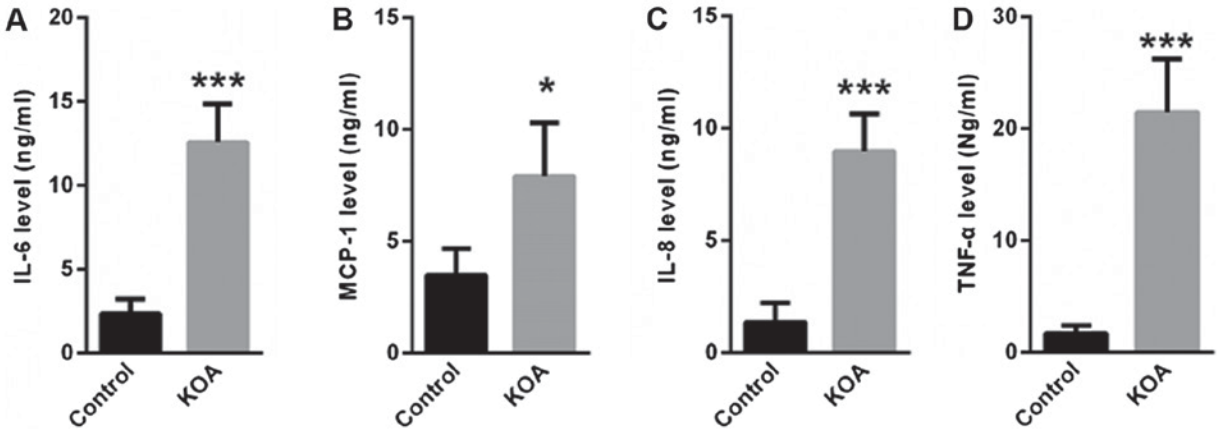

Figure 1. Inflammatory factors were increased in the vastus lateralis muscles of KOA patients. ELISA showed that the levels of IL-6 (A), MCP-1 (B), IL-8 (C) and TNFα (D) were significantly increased in the muscle tissues of KOA patients compared with those of control individuals. *P<0.05 and ***P<0.001 vs. control. KOA, knee osteoarthritis; ELISA, enzyme-linked immunosorbent assay; TNFα, tumor necrosis factor α.

temperature for 20 min, embedded in paraffin and cut into 5-μm thick sections, followed by deparaffinizition, descending alcohol series of rehydration, and microwave-heating in sodium citrate buffer (Solarbio Science & Technology Co., Ltd.) at 100˚C for 30 min for antigen retrieval. Sections were subsequently incubated with 0.3% hydrogen peroxide/phosphate-buffered saline for 30 min. The sections were incubated with a primary anti-p-p65 antibody (#3033; Cell Signaling Technology, Inc.,) or anti-IκBα (#4812, Cell Signaling Technology, Inc.) at a 1:50 dilution and 4˚C overnight. Detection of the primary antibody was performed via incubation with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (ZDR-5036, Zhongshan Gold Bridge Biological Technology Co.,) for 1 h at room temperature and visualized with a 3,3'-Diaminobenzidine substrate. Stained cells were counted in 5 random fields using light microscopy (magnification, 40x, Olympus CK40; Olympus Corporation, Tokyo, Japan).

Immunofluorescence. Primary human skeletal muscle cells (~1x10^{6}) cells were cultured in a 6-well plate for 24 h with glass coverslips. After that, the cells were transfected with Ad-NC or Ad-USF1 for 48 h. Then, the cells on the coverslips were fixed in 4% paraformaldehyde for 30 min at room temperature. The samples were washed three times in PBS for 5 min and fluorescence intensity was examined using a fluorescence microscope (Olympus Corporation) at a magnification of x100.

Statistical analysis. Data are presented as the mean ± standard deviation. To compare the two groups, two-tailed unpaired Student's t-test was performed. For multiple group comparisons, one-way analysis of variance followed by Tukey's post hoc test were used. Statistical tests were performed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Decreased muscle strength was identified in the muscle tissues of KOA patients. The basic physical characteristics are shown in Table I. No significance was found in the age, height, weight and BMI of patients with KOA or healthy controls. By contrast, lower muscle strength was identified in KOA patients than in healthy controls (Table I).

Increased inflammatory factors in the vastus lateralis muscle tissues of KOA patients. Next, we determined the inflammatory factors in the vastus lateralis muscle tissues of KOA patients and control individuals. ELISA showed that the levels of IL-6, MCP-1, IL-8 and TNFα were significantly increased in the muscle tissues of KOA patients compared with control individuals (Fig. 1).

NF-κB signaling is activated in the vastus lateralis muscle tissues of KOA patients. p65 NF-κB signaling is a key signaling pathway that upregulates cytokine gene expression, including TNFα, IL-1β, IL-6, and MCP-1, in muscle tissues (7,9). Thus, we analyzed p65 NF-κB activation in the muscle tissues of KOA patients and control individuals. Western blot assays indicated that p65 NF-κB was significantly increased in the muscle tissues of KOA patients compared with control individuals, while IκBα, an inhibitor of NF-κB, was shown to be decreased in the muscle tissues of KOA patients (Fig. 2). We also analyzed histological changes of p-p65 and IκBα in skeletal muscle. In line with the findings of western blot, p-p65 was found to be enhanced in the muscle tissues of KOA patients compared with control individuals, but IκBα was decreased in the muscle tissues of KOA patients (Fig. 2B and C).

Upregulation of USF1 in the vastus lateralis muscle tissues of KOA patients. Furthermore, we evaluated the expression of USF1 in muscle tissues of KOA patients. Compared with control individuals, the protein levels of USF1 were significantly enhanced in the muscle tissues of KOA patients (Fig. 3).

USF1 activates NF-κB signaling in primary human skeletal muscle cells. To further explore whether USF1 activates NF-κB signaling in primary human skeletal muscle cells, adenovirus vectors overexpressing USF1 or NC were transfected into primary human skeletal muscle cells for 48 h.

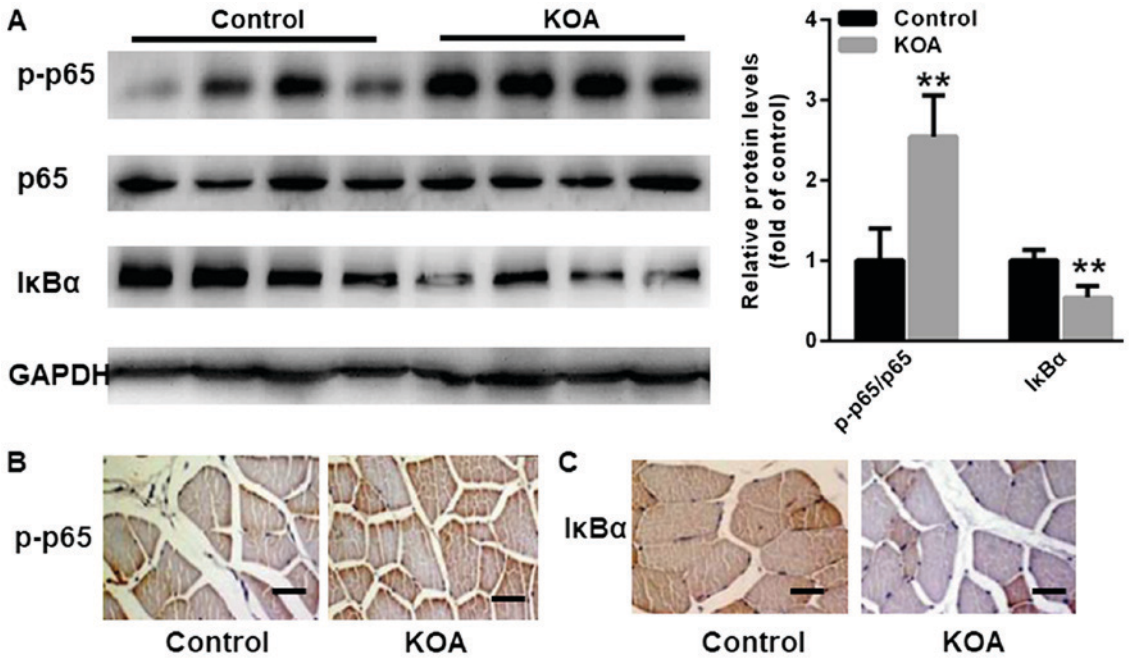

Figure 2. NF-κB signaling was activated in the vastus lateralis muscle tissues of KOA patients compared with those of control individuals. (A) Western blot assay showed that the phosphorylation of p65 was increased, but the expression of IκBα was decreased in muscle tissues of KOA patients. IHC staining of p-p65 (B) and IκBα (C) in the muscle tissues of KOA patients compared with control. Bar represent 50 μm, x100; **P<0.01 vs. control. KOA, knee osteoarthritis.

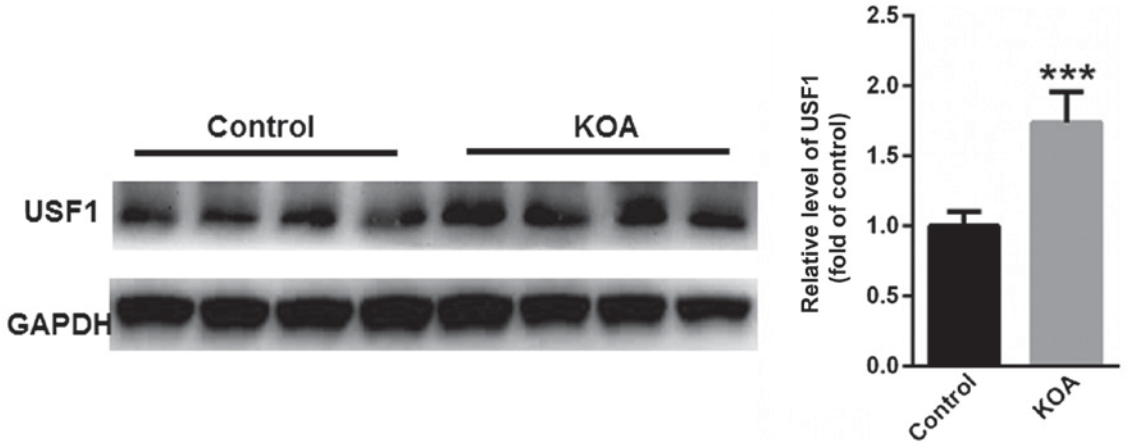

Figure 3. Western blot assays indicated that USF1 was increased in the vastus lateralis muscle tissues of KOA patients compared with control individuals. ***P<0.001 vs. control. USF1, upstream stimulating factor 1; KOA, knee osteoarthritis.

As shown in Fig. 4A, the transfection efficiency was similar between Ad-NC or Ad-USF1 in in primary human skeletal muscle cells. Western blot assays indicated that overexpression of USF1 significantly induced the transcription of p65 NF-κB signaling (Fig. 4B). Moreover, an ELISA assay revealed upregulation of inflammatory factors, including TNFα (Fig. 4C), IL-8 (Fig. 4D), IL-6 (Fig. 4E) and MCP-1 (Fig. 4F).

Discussion

The progression of KOA is accompanied by injury of the entire joint structure and increased inflammation in the joint (20,21). Impaired muscle strength and dysfunction are common features in the affected legs and likely decrease the quality of life among patients with knee OA (22,23). Thus, it is important to improve inflammation-induced impairments in muscle strength among patients with KOA.

Muscle weakness is a typical characteristic in patients with KOA (24,25). In accordance with previous findings, our data showed that muscle strength was significantly decreased in KOA patients compared with controls. Increasing evidence has indicated that inflammatory responses can induce significant changes in the cellular microenvironment that then result in the survival, repair and maintenance of muscle cells (21,26). In KOA patients, it has been reported that the levels of pro-inflammatory cytokines are significantly increased and muscle mass is obviously decreased (27,28). Our data showed that several inflammatory factors, including TNFα, IL-8, IL-6 and MCP-1, were associated with reduced muscle strength in KOA patients. These observations suggest

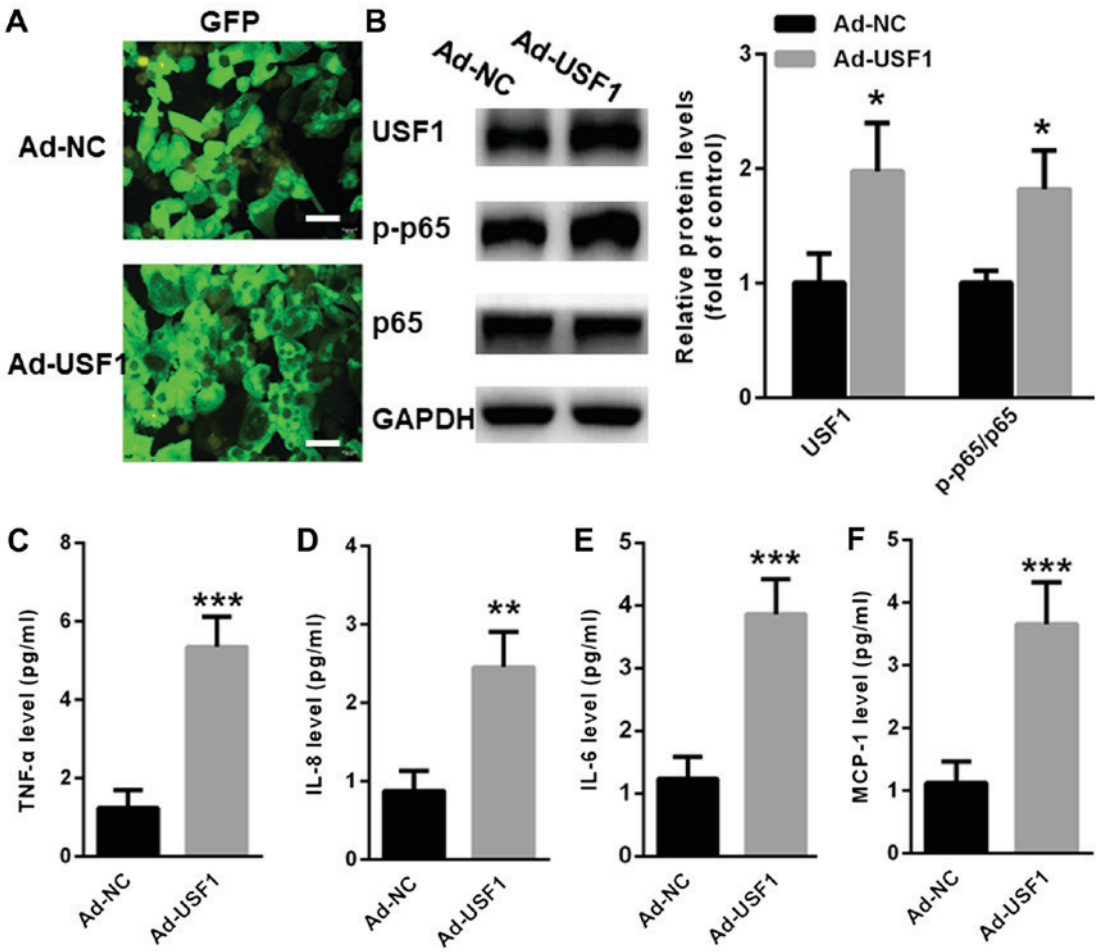

Figure 4. USF1 activates NF-κB signaling in primary human skeletal muscle cells. (A) Fluorescence assay showed the transfection efficiency of Ad-NC or Ad-USF1 in primary human skeletal muscle cells. Scale bar, 20 μm. (B) Western blot assays indicated that the overexpression of USF1 significantly induced the transcription of p65 NF-κB signaling. An ELISA assay revealed upregulation of inflammatory factors, including TNFα (C), IL-8 (D), IL-6 (E) and MCP-1 (F). *P<0.05, **P<0.01 and ***P<0.001 vs. control. USF1, upstream stimulating factor 1; ELISA, enzyme-linked immunosorbent assay; TNFα, tumor necrosis factor α.

that the enhancement of proinflammatory molecules within the muscle tissues may impair physical function among KOA patients.

Increased NF-κB activity in injured muscle fibers is widely reported to diminish the myogenic potential of their associated satellite cells (29). Furthermore, the p105/p50 subunit in NF-κB knockout mice has been demonstrated to be partially resistant to muscle atrophy (30). Thus, we evaluated the activation of NF-κB signaling in the vastus lateralis muscle tissues of KOA patients compared with controls. Not surprisingly, NF-κB signaling was significantly activated in the muscle tissues of KOA patients compared with control individuals. Thus, it is of great importance to elucidate the underlying cellular mechanisms that regulate inflammatory signaling in the muscle tissues of KOA patients, thereby providing a novel therapeutic method for treating KOA.

In skeletal muscle, the transcription of the mouse type Iα (RIα) subunit of the cAMP-dependent protein kinase begins at the alternative noncoding first exons 1a and 1b (31). A previous study has indicated that the regulation of the promoter upstream of exon 1a (Pa) depends on two adjacent E boxes (E1 and E2) in intact muscle (31). More importantly, USF1 is an important transcription factor that binds the E-box elements in the promoter region of muscle-specific genes (32,33). However, the expression pattern of USF1 in the muscle tissues of KOA patients has never been reported. For the first time, we showed that USF1 was increased in the muscle tissues of KOA patients compared with control. More importantly, overexpression of USF1 in primary human skeletal muscle cells significantly increased the activation of NF-κB signaling as well as the levels of pro-inflammatory factors. Thus, our data showed that USF1 activated NF-κB signaling in muscle tissues of KOA patients, which was then involved in inflammation-induced muscle weakness.

To our knowledge, this is the first study to explore a relationship between USF1 and NF-κB activation-induced inflammatory responses in muscle tissues of KOA patients, with findings aimed at improving the inflammatory response and preventing physical disability. However, we have to admit that some limitations exist in the current study. For instance, how the expression of USF1 was upregulated in the muscle tissues of KOA patients. In addition, whether other signaling pathways are involved in the correlation between USF1 and inflammation response in muscle tissues of KOA patients deserves further exploration. In the future, we will carry out deep research on the above questions thereby fully elucidating the underlying mechanism by which USF1 is modulated in the progression of KOA.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XS performed the experiments and analyzed the data. HL, BL, ZY, WW, HL, JS and SL performed the IHC staining and western blot experiments. MZ designed the experiments, analyzed the data and gave final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Hongqi Hospital Affiliated with Mudanjiang Medical University (Mudanjiang City, China) and all the patients have provided written informed consent for this study.

Patient consent for publication

Informed consent for participation in the study or use of their tissue was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

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